BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 9/22, C07K 3/20
A61K 37/54

A1

(11) International Publication Number: **WO 93/25670**

(43) International Publication Date: 23 December 1993 (23.12.93)

(21) International Application Number: PCT/US93/05136

(22) International Filing Date: 28 May 1993 (28.05.93)

(30) Priority data:
07/895,300 8 June 1992 (08.06.92) US

(71) Applicant: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).

(72) Inventors: FRENZ, John ; 256 Elder Avenue, Millbrae, CA 94030 (US). SHIRE, Steven, J. ; 2417 Lincoln Avenue, Belmont, CA 94002 (US). SLIWKOWSKI, Mary, B. ; 10 Bellflower Lane, San Carlos, CA 94070 (US).

(74) Agents: JOHNSTON, Sean, A. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).

(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: PURIFIED FORMS OF DNASE

(57) Abstract

The present invention provides the identification and characterization of two components of a recombinant preparation of DNase. These components are the purified deamidated and non-deamidated human DNases. Taught herein are the separation of these components and the use of the non-deamidated species as a pharmaceutical per se, and in particular in compositions wherein the species is disclosed within a plastic vial, for use in administering to patients suffering from pulmonary distress.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**MR**	Mauritania
AU	Australia	**GA**	Gabon	**MW**	Malawi
BB	Barbados	**GB**	United Kingdom	**NL**	Netherlands
BE	Belgium	**GN**	Guinea	**NO**	Norway
BF	Burkina Faso	**GR**	Greece	**NZ**	New Zealand
BG	Bulgaria	**HU**	Hungary	**PL**	Poland
BJ	Benin	**IE**	Ireland	**PT**	Portugal
BR	Brazil	**IT**	Italy	**RO**	Romania
CA	Canada	**JP**	Japan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SK**	Slovak Republic
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SN**	Senegal
CM	Cameroon	**LI**	Liechtenstein	**SU**	Soviet Union
CS	Czechoslovakia	**LK**	Sri Lanka	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**MC**	Monaco	**UA**	Ukraine
DK	Denmark	**MG**	Madagascar	**US**	United States of America
ES	Spain	**ML**	Mali	**VN**	Viet Nam
FI	Finland	**MN**	Mongolia		

PURIFIED FORMS OF DNASE

Related Patent Applications

The present application is related in subject matter to the disclosure contained in International Patent Application Publication No. WO 90/07572. The content of this prior application is hereby expressly incorporated by reference herein.

Field of the Invention

The present invention is related to results obtained from research on deoxyribonuclease (DNase), a phosphodiesterase that is capable of hydrolyzing polydeoxyribonucleic acid. It relates generally to the separation of several forms of said DNase, to these forms per se, to pharmaceutical compositions by which their utility can be exploited clinically, and to methods of using these DNases and compositions thereof.

Background of the Invention

DNase is a phosphodiesterase capable of hydrolyzing polydeoxyribonucleic acid. DNase has been purified from various species to various degrees. The complete amino acid sequence for a mammalian DNase was first made available in 1973. See e.g., Liao, et al., J. Biol. Chem. 248:1489 (1973).

DNase has a number of known utilities and has been used for therapeutic purposes. Its principal therapeutic use has been to reduce the viscoelasticity of pulmonary secretions in such diseases as pneumonia and cystic fibrosis, thereby aiding in the clearing of respiratory airways. See e.g., Lourenco, et al., Arch. Intern. Med. 142:2299 (1982); Shak, et al., Proc. Nat. Acad. Sci. 87:9188 (1990); Hubbard, et al., New Engl. J. Med. 326:812 (1992).

DNA encoding human DNase I has been isolated and sequenced and that DNA has been expressed in recombinant host cells, thereby enabling the production of human DNase in commercially useful quantities. See e.g., Shak, et al., Proc. Nat. Acad. Sci. 87:9188-9192 (1990). Recombinant human DNase (rhDNase) has been found to be useful clinically, especially in purified form such that the DNase is free from proteases and other proteins with which it is ordinarily associated in nature. See e.g., Hubbard, et al., New Engl. J. Med. 326:812 (1992).

The means and methods by which human DNase can be obtained in pharmaceutically effective form is described in the patent applications cited above. Various specific methods for the purification of DNase are known in the art. See e.g., Khouw, et al., U.S. Patent No. 4,065,355 (issued 27 December 1977); Markey, FEBS

Letters 167:155 (1984); Nefsky, et al., Eur. J. Biochem. 179:215 (1989).

Although it was not appreciated at the time the above-referenced patent applications were filed, the DNase product obtained from cultures of recombinant host cells typically comprises a mixture of deamidated and non-deamidated forms of DNase. The existence of deamidated forms of DNase remained unappreciated notwithstanding that the phenomenon of deamidation of asparagine and glutamine residues in some proteins is known. See e.g., Kipper et al., Ann. Rev. Physiol. 50:333 (1988); Kossiakoff, Science 240:191 (1988); Bradbury et al., Trends in Biochem. Sci. 16:112 (1991); and Wright, Protein Engineering 4:283 (1991).

The present invention is predicated upon the previously unappreciated fact that recombinant human DNase may exist as a mixture of deamidated and non-deamidated forms. Using the methods of the present invention, it has been found that deamidated human DNase is less active enzymatically than non-deamidated human DNase. Thus, the presence of the deamidated DNase and non-deamidated DNase together in a mixture, and the potential for further deamidation occurring, such as has been found to occur upon in vitro storage of preparations of human DNase, may complicate efforts to provide consistent uniformity in a DNase product being administered clinically. Therefore, as the existence and characteristics of deamidated DNase were not known prior to the present invention, the methods for identifying deamidated DNase and separating it from preparations of DNase in which it may be found were unobvious at the time this invention was made.

Summary of the Invention

The present invention is directed to processes for separating the deamidated and non-deamidated human DNase forms from a mixture thereof. This process in preferred embodiments comprises subjecting the mixture to chromatography using a resin, or other support medium, having bound thereto a cationic polymer such as heparin or a non-hydrolyzable deoxyribonucleic acid (DNA) analog, or chromatography using a so-called tentacle cation exchange resin. The present invention also is directed to the use of those chromatographic methods with non-human DNases, such as bovine DNase.

The present invention also is directed to deamidated human DNase as a purified product, substantially free of non-deamidated human DNase.

The present invention also is directed to non-deamidated human DNase as a purified product, substantially free of deamidated human DNase. It has been found herein that purified non-deamidated human DNase is fully enzymatically active as compared with deamidated human DNase.

The present invention also is directed to pharmaceutical compositions consisting of either purified deamidated human DNase or purified non-deamidated human DNase as the active principle, optionally together with a pharmaceutically acceptable excipient.

The present invention also is directed to a method comprising administering a therapeutically effective amount of purified deamidated human DNase or purified non-deamidated human DNase for the treatment of a patient, for example those having an accumulation of viscous, DNA-containing material. The administration of such purified DNases preferably is effected by direct inhalation into the lungs.

The present invention is particularly directed to a method of treating a patient having a pulmonary disease such as chronic bronchitis, cystic fibrosis, or emphysema, that comprises administering a therapeutically effective amount of purified non-deamidated human DNase, preferably directly into the airway passages.

The present invention also is directed to pharmaceutical compositions comprising non-deamidated human DNase that are disposed within a plastic vial, optionally in the presence of a pharmaceutically acceptable excipient.

Brief Description of the Drawings

Figure 1 depicts the amino acid (SEQ.ID.NO. 1) and DNA sequences (SEQ.ID.NO. 2) of human DNase I. The native signal sequence is underlined, the potential initiation codons are circled, and the mature sequence is bracketed.

Figure 2 depicts the correlation between enzymatic activity and extent of deamidation of samples of human DNase. Specific activity was determined by normalizing the DNase activity as determined by a methyl green (MG) assay (in concentration units relative to a standard curve) to the DNase concentration measured by an enzyme-linked immunoabsorbent assay (ELISA). Percent deamidation was determined by tryptic mapping. "Day of Harvest" samples of human DNase were purified from a culture of recombinant Chinese hamster ovary (CHO) cells expressing DNA encoding human DNase I. Such samples were taken at 3, 5, 7, 9, 11, 13, and 20 days after the culture was started. "High pH" samples were day 13 samples of purified DNase that were incubated in vitro for two days at pH 8 at 37°. "Stability" samples were day 13 samples of purified DNase that were stored in vitro at 5°, 25°, or 37° C for various periods of time.

Figure 3 is an example of a tryptic map of DNase employed for determination of the extent of deamidation. The sample shown here is 65% deamidated DNase. "mAU" indicates milli-absorbance units at 214 nM.

Figure 4 is a schematic representation of the deamidation of the asparagine residue at amino acid position 74 (Asn-74) in native human

DNase. Deamidation converts the Asn-74 to either an aspartic acid (Asp) or an iso-aspartate (iso-Asp) residue. Each of the three forms of DNase yields, on digestion with trypsin, a pair of peptides that indicates the identity of the particular form of DNase.

Figure 5 is a chromatogram of a human DNase sample fractionated on a tentacle cation exchange (TCX) column. The sample shown is 67% deamidated DNase.

Figure 6 shows tryptic maps of the two peak fractions from the TCX separation shown in Figure 5. The absence of tryptic peptide T6-7 from the map of the Peak 2 digest indicates the absence of deamidated DNase.

Figure 7 shows chromatograms of several human DNase samples fractionated on a TCX column. The sample designated "M1-28 STD." is a preparation of human DNase obtained from a culture of Chinese hamster ovary (CHO) cells transformed with DNA encoding native human DNase I. The sample designated "DNase ASP Mutant" is DNase having an aspartic acid residue (rather than an asparagine residue) at amino acid position 74, and which thus has the same amino acid sequence as the Asp form of deamidated DNase shown in Figure 4. The DNase ASP Mutant was obtained from a culture of cells transformed with DNA encoding that mutant form of human DNase. The DNA encoding the DNase ASP Mutant was prepared by site-directed mutagenesis of DNA encoding native human DNase. Comparison of the chromatograms shows that one of the forms of human DNase in the M1-28 STD. elutes from the TCX column at the same position as the DNase Asp Mutant.

Figure 8 shows chromatograms of several human DNase samples fractionated on a TSK-Heparin column (Toso Haas, Montgomeryville, Pennsylvania). The sample designated "12K #8" is a preparation of human DNase obtained from a culture of Chinese hamster ovary (CHO) cells transformed with DNA encoding native human DNase I. The sample designated "Deamidated Standard" is purified deamidated human DNase. The sample designated "Non-deamidated standard" refers to purified non-deamidated human DNase.. Purified deamidated human DNase and purified non-deamidated human DNase were prepared by TCX chromatography.

Figure 9 shows chromatograms of several human DNase samples fractionated on an immobilized DNA analog column. The sample designated "M1-28" is a preparation of human DNase obtained from a culture of Chinese hamster ovary (CHO) cells transformed with DNA encoding native human DNase I. The sample designated "Deamidated Standard" is purified deamidated human DNase. The sample designated "Non-deamidated standard" refers to purified non-deamidated human DNase. Purified deamidated human DNase and purified non-deamidated human DNase was prepared by TCX chromatography.. The sample designated

"DNase ASP Mutant" is DNase having an aspartic acid residue (rather than an asparagine residue) at amino acid position 74.

Detailed Description

A. Definitions

By the term "human DNase" herein is meant a polypeptide having the amino acid sequence of human mature DNase I set forth in Figure 1 as well as amino acid sequence variants thereof (including allelic variants) that are enzymatically active in hydrolyzing DNA. Thus, the term "human DNase" herein denotes a broad definition of those materials disclosed and prepared in the patent applications described above.

The term "human DNase" necessarily embraces native mature human DNase having an asparagine (Asn) residue at amino acid position 74 of the polypeptide. That asparagine has been found herein to be susceptible to deamidation, which deamidation may produce a mixture of deamidated and non-deamidated forms of human DNase. Instead of the Asn residue at amino acid position 74, deamidated DNase has an aspartic acid (Asp) or an iso-aspartate (iso-Asp) residue (see Figure 4).

The term "deamidated human DNase" as used herein thus means human DNase that is deamidated at the asparagine residue that occurs at position 74 in the amino acid sequence of native mature human DNase. It has been found that deamidated human DNase may arise during the production of human DNase by recombinant means, and may be found in preparations of human DNase obtained from recombinant host cells. Additionally, deamidated human DNase may arise upon in vitro storage of non-deamidated human DNase.

Although the asparagine residue at amino acid position 7 in the amino acid sequence of native mature human DNase also may be deamidated (in addition to the asparagine residue at amino acid position 74), such doubly deamidated DNase has been found to be enzymatically inactive.

The term "mixture" as used herein in reference to preparations of human DNase means the presence of both deamidated and non-deamidated forms of human DNase. It has been found, for example, that in preparations of human DNase obtained from recombinant expression, as much as about 50% to 80% or more of the human DNase is deamidated.

The term "purified deamidated human DNase" as used herein means deamidated human DNase that is substantially free of non-deamidated human DNase. In other words, non-deamidated human DNase will comprise less than about 10%, preferably less than about 5%, and most preferably less than about 1% by weight of the total DNase in the purified deamidated human DNase composition.

The term "purified non-deamidated human DNase" as used herein means non-deamidated human DNase that is substantially free of deamidated human DNase. In other words, deamidated human DNase will comprise less than about 25%, preferably less than about 5%, and most preferably less than about 1% by weight of the total DNase in the purified non-deamidated human DNase composition.

By the term "excipient" herein is meant a pharmaceutically acceptable material that is employed together with DNase for the proper and successful administration of the DNase to a patient. Suitable excipients are well known in the art, and are described, for example, in the Physicians Desk Reference, the Merck Index, and Remington's Pharmaceutical Sciences.

A preferred formulation for human DNase is a buffered or unbuffered aqueous solution, and preferably is an isotonic salt solution such as 150 mM sodium chloride containing 1.0 mM calcium chloride at pH 7. These solutions are particularly adaptable for use in commercially-available nebulizers including jet nebulizers and ultrasonic nebulizers useful for administration, for example directly into the airways or lungs of an affected patient. Reference is made to the above-identified patent applications for further detail concerning how human DNase can be formulated and administered for effective use.

By the term "therapeutically effective amount" herein, is meant dosages of from about 1 μg to about 100 mg of human DNase per kilogram of body weight of the patient, administered within pharmaceutical compositions, as described herein. The therapeutically effective amount of human DNase will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. In view of the differences in enzymatic activity between deamidated and non-deamidated DNases described herein, it may be that the amount of purified non-deamidated DNase required to achieve a therapeutic effect will be less than the amount of purified deamidated human DNase or a mixture of the two forms necessary to achieve the same effect under the same conditions.

The purified DNases hereof, particularly the non-deamidated form, are employed for enzymatic alteration of the viscoelasticity of mucous. Such purified human DNases are particularly useful for the treatment of patients with pulmonary disease who have abnormal viscous, purulent secretions and conditions such as acute or chronic bronchial pulmonary disease, including infectious pneumonia, bronchitis or tracheobronchitis, bronchiectasis, cystic fibrosis, asthma, tuberculosis, and fungal infections. For such therapies, a solution or finely divided dry preparation of purified deamidated

human DNase or purified non-deamidated human DNase is instilled in conventional fashion into the bronchi, for example by aerosolization.

B. Preferred Embodiments

After the successful cloning and expression of human DNase in recombinant host cells, it was discovered after substantial research that the DNase product obtained from such recombinant expression typically existed as a mixture of as then yet undefined components. In particular, isoelectric focusing (IEF) analysis of human DNase purified from cultures of recombinant Chinese hamster ovary (CHO) cells revealed a complex pattern of DNase species. The various DNase species were determined to result from several post-translational modifications of the DNase, including deamidation.

Two assays were used to determine the presence and extent of deamidated DNase in such preparations. One method involved tryptic digestion of the starting preparation of DNase and analysis of the resulting peptides by reverse phase HPLC. In this method, the amount of deamidated DNase in the starting preparation was determined by measuring the quantities of six deamidation-indicating tryptic peptides.

The other method involved chromatography of the starting preparation of DNase on a tentacle cation exchange (TCX) column. It was discovered that the TCX column is capable of resolving deamidated human DNase and non-deamidated human DNase, such that each form of DNase could be effectively separated from the other, and obtained in purified form. In this method, the amount of deamidated and non-deamidated DNase in the starting preparation was determined by measuring on chromatograms the peak areas corresponding to the separated forms of DNase.

Although these two methods are about equally effective in determining and quantitating deamidated DNase, the TCX method is especially efficient, requiring far less time and labor than the other method. Moreover, TCX chromatography provides a means for separating deamidated and non-deamidated forms of DNase, whereas conventional cation exchange resins and various other chromatography resins that were analyzed were not capable of such separation.

The general principles of TCX chromatography have been described, for example, by Miller, J. Chromatography 510:133 (1990); Janzen et al., J. Chromatography 522:77 (1990); and Hearn et al., J. Chromatography 548:117 (1991). Without limiting the invention to any particular mechanism or theory of operation, it is believed that the Asn-74 residue in human DNase that is susceptible to deamidation is located within the DNA-binding groove of the enzyme, by analogy to the known crystal structure of bovine DNase. The DNA-binding groove contains basic amino acid residues (in order to bind DNA) and this

groove apparently is accessible to the ligands of the tentacle cation exchange resin but not to the much shorter ligands of conventional cation exchange resins. Presumably the ligands of the tentacle cation exchange resin mimic natural nucleic acid substrates. Therefore, it is expected that tentacle action exchange chromatography will be useful for the purification of other nucleases, such as ribonuclease (RNase) or restriction endonucleases, as well as DNA binding proteins.

Alternatively, the separation of deamidated and non-deamidated forms of DNase may be accomplished by chromatography using a resin or other support matrix containing covalently bound cationic polymers such as heparin or a synthetic non-hydrolyzable DNA analog. Immobilized heparin chromatography columns are commercially available (for example, from Toso Haas Co., Montgomeryville, Pennsylvania). Non-hydrolyzable DNA analogs have been described, for example, by Spitzer et al., Nuc. Acid. Res. 16:11691 (1988). An immobilized non-hydrolyzable DNA analog column is conveniently prepared by synthesizing such a DNA analog with an amino acid group at the 3'-end of one or both of its complementary strands. The amino group is then available for coupling to an epoxy-activated column, as described, for example, in literature published by Rainin Biochemical LC Products (Woburn, Massachusetts).

Following the successful separation of deamidated and non-deamidated human DNases according to the methods of the present invention, it was found that deamidated human DNase has diminished enzymatic activity as compared to non-deamidated human DNase, as determined by a methyl green (MG) assay. Kurnick, Arch. Biochem. 29:41 (1950). It was found that deamidated human DNase exhibits just over half of the enzymatic activity of non-deamidated human DNase. Thus, by combining the purified deamidated DNases and the purified non-deamidated DNase of the present invention in various proportions, it is possible to prepare pharmaceutical compositions of human DNase having any desired specific activity in the range between the specific activities of the individual components, as may be optimal for treating particular disorders.

The following examples are offered by way of illustration only and are not intended to limit the invention in any manner.

C. Examples

1. Tryptic Mapping.

The procedure used for tryptic mapping of human DNase is summarized as follows:

Step 1. Bring concentration of 1 mg sample of DNase to 4 mg/ml by concentration on Amicon Centricon-10 device or by dilution with excipient. Final volume: 250 µl.

Step 2. Add 250 µl of pretreatment buffer (40 mM BisTris, 10 mM EGTA, pH 6.0) to sample. Incubate 1 hour at 37°.

Step 3. Buffer exchange sample into digest buffer (100 mM Tris, pH 8) using Pharmacia NAP-5 column. Final volume: 1 ml.

Step 4. Add 10 µl trypsin solution (1 mg/ml trypsin, 1 mM HCl) to sample and incubate 2 hours at 37°.

Step 5. Add second 10 µl aliquot of trypsin solution to sample and incubate additional 2 hours at 37°.

Step 6. Stop digestion by addition of 6 µl trifluoroacetic acid (TFA). Store samples at or below 5° until chromatographed.

Step 7. Separate the peptide mixture by HPLC under the following conditions:

Column: Nucleosil C18, 5 µm, 100 Å, 2.0 x 150 mm (Alltech, Co., Deerfield, Illinois).
Column temperature: 40°.
Eluent A: 0.12% TFA in water.
Eluent B: 0.10% TFA in acetonitrile.
Gradient profile:

Time (min)	%A	%B
0	100	0
5	100	0
65	40	60
69	5	95
70	5	95

Flow rate: 0.25 ml/min. Sample injection volume: 250µl.
Post-run column reequilibration time at 100% A: 20 min.
Autosampler compartment temperature: 5°.
Detection: Absorbance at 214 and 280 nm.

Step 8. Identify T7, (D)T7, T7-8, (D)T7-8, T6-7-8, and T6-7 tryptic peptides by retention time comparison with standard.

Step 9. Integrate chromatogram obtained at 280 nm. Check quality of integration by inspection of baseline and separation of closely eluting peaks. Special attention must be paid to the early-eluting T7 and (D)T7 peptides that may not be well-resolved.

Step 10. Normalize peak areas of the six reporter peptides to tyrosine content. Peptides T7, (D)T7, T7-8, and (D)T7-8 each contain a single Tyr residue, while T6-7-8 and T6-7 contain three Tyr residues. Calculate the proportion of deamidated species based on the normalized peak areas of (D)T7, (D)T7-8, T6-7-8, and T6-7 relative to the total normalized peak areas of the six peptides.

One milligram of DNase in a volume of 250 µl is required in order to accurately carry out the tryptic mapping method for determination of deamidated DNase according to the procedure outlined above. Hence, the initial sample preparation for this method requires either

concentration or dilution of the sample to achieve that result. DNase in the presence of calcium is highly resistant to proteases, including trypsin. Therefore the next step in the procedure is to partially remove calcium ions by treatment with [ethylene bis(oxyethylenenitrilo)] tetraacetic acid (EGTA). Over-treatment with EGTA can denature and aggregate DNase, so this step must be performed with care. The EGTA-treated sample in a volume of 0.5 ml is then exchanged into 1 ml of the digest buffer, trypsin added, and the sample incubated at 37° for two hours. A second aliquot of trypsin is then added and the sample incubated an additional two hours. Digestion is stopped by acidification, and the sample is either stored for later analysis or loaded on the HPLC column directly.

250 µl (250 µg) of the peptide mixture resulting from the tryptic digestion is separated on a reversed phase HPLC column according to the conditions outlined above. A typical tryptic map of human DNase is shown in Figure 3. HPLC was performed with a Hewlett-Packard model 1090M HPLC. The column effluent was monitored simultaneously at 214 and 280 nm by the diode array detector that is a feature of this instrument. Since the early portion of the peptide map is critical to the quantitation of deamidated DNase, as described below, other instruments with larger gradient delay and other extra-column volumes may not be suited to this analysis. Each analysis by this procedure requires 70 minutes for the gradient separation and 20 minutes to re-equilibrate the column for a total HPLC turnaround time of 90 minutes. The rationale and approach to peak integration for determination of deamidated DNase in a sample are described below.

Deamidation of human DNase occurs at least at the asparagine residue that is present at amino acid position 74 (Asn-74) in native mature human DNase. Asn-74 is on the C-terminal side of a tryptic cleavage site at the arginine residue at amino acid position 73 (Arg-73), as seen in the list of expected tryptic peptides of human DNase shown in Table I.

TABLE I. PEPTIDES EXPECTED TO BE PRODUCED UPON DIGESTION OF NATIVE MATURE HUMAN DNASE WITH TRYPSIN.

ID	Residues	Amino Acid Sequence of Peptide
T1	1-2	LK
T2	3-15	IAAFNIQTFGETK (SEQ.ID.NO. 3)
T3	16-31	MSNATLVSYIVQILSR (SEQ.ID.NO. 4)
T4	32-41	YDIALVQEVR (SEQ.ID.NO. 5)
T5	42-50	DSHLTAVGK (SEQ.ID.NO. 6)
T6	51-73	LLDNLNQDAPDTYHYVVSEPLGR (SEQ.ID.NO. 7)
T7	74-77	NSYK (SEQ.ID.NO. 8)
T8	78-79	ER
T9	80-111	YLFVYRPDQVSAVDSYYYDDGCEPCGNDTFNR (SEQ.ID.NO. 9)
T10	112-117	EPAIVR (SEQ.ID.NO. 10)
T11	118-121	FFSR (SEQ.ID.NO. 11)
T12	122-126	FTEVR (SEQ.ID.NO. 12)
T13	127-157	EFAIVPLHAAPGDAVAEIDALYDVYLDVQEK (SEQ.ID.NO.13)
T14	158-185	WGLEDVMLMGDFNAGCSYVRPSQWSSIR (SEQ.ID.NO. 14)
T15	186-213	LWTSPTFQWLIPDSADTTATPTHCAYDR (SEQ.ID.NO. 15)
T16	214-222	IVVAGMLLR (SEQ.ID.NO. 16)
T17	223-260	GAVVPDSALPFNFQAAYGLSDQLAQAISDHYPVEVMLK (SEQ.ID.NO. 17)

Instead of the Asn (single letter designation "N") residue at residue 74 in native, non-deamidated human DNase, deamidated human DNase has either an Asp or iso-Asp residue, as shown in Figure 4. Iso-Asp is an isomeric, beta-amino acid form of aspartic acid. The peptide bond between Arg-73 and iso-Asp is resistant to cleavage by trypsin, so deamidated human DNase yields a characteristic tryptic peptide containing residues 51-77 and called T6-7 since it is the conjoined peptides T6 and T7. Under conditions employed for tryptic mapping, the Arg-73-Asn-74 peptide bond in non-deamidated human DNase and the Arg-73-Asp-74 peptide bond in the Asp form of deamidated human DNase are cleaved by trypsin. Hence, non-deamidated DNase is indicated in the tryptic map by the presence of T7 peptide shown in Table I, while the Asp-74 form of deamidated human DNase is indicated in the tryptic map by the presence of the deamidated T7 peptide, called (D)T7. These three reporter peptides are labelled in Figure 3. Unfortunately, trypsin only partially cleaves the peptide bond at the C-terminal side of T7, between residues 77 and 78, so that each of the reporter peptides T7, (D)T7 and T6-7 has a T8-conjugate, T7-8, (D)T7-8 and T6-7-8, respectively. These six reporter peptides must therefore be accounted for in order to quantitate deamidated human DNase by the tryptic mapping method.

In principle, the (D)T7, (D)T7-8, T6-7 and T6-7-8 peptides represent deamidated human DNase and the T7 and T7-8 peptides represent non-deamidated human DNase and knowledge of the relative proportions of these peptides permits a straightforward calculation of the extent of deamidation in a preparation of DNase. In order to calculate the fraction of the sample that is deamidated DNase, knowledge of the molar ratios of deamidated and non-deamidated species is required, but the

There are two additional problems in the tryptic mapping procedure that must be overcome: one chromatographic problem and one detection problem. The chromatographic problem is that the T2 peptide coelutes with T6-7, and so impedes the integration of an accurate peak area of this deamidation-indicating peptide. This problem can be overcome by integration of the chromatogram obtained at 280 nm, since all six of the relevant peptides have at least one tyrosine (Tyr) residue, and so absorb strongly at 280 nm, while T2 contains no Tyr or tryptophan (Trp) residues and thus absorbs negligibly at this wavelength. The detection problem is that the T6-7 and T6-7-8 peptides each contain three Tyr residues while the other four peptides each contain only one. Thus the T6-containing peptides have a higher molar absorptivity than do the peptides that contain only T7, and a simple comparison of peak areas would tend to overestimate the content of deamidated species in a sample. This problem is overcome by normalizing the peak areas of the six peptides to the number of Tyr residues in the peptide. Normalizing the peak areas in this manner implies that all tyrosine residues in each of the peptides is in an equivalent chemical environment, which is probably a good assumption for relatively small peptides such as considered here. Upon normalization, the corrected peak areas for deamidated and non-deamidated peptides can be compared to arrive at an estimate of the content of deamidated DNase in a sample.

2. Tentacle Cation Exchange Chromatography.

Tentacle cation exchange (TCX) resins, unlike conventional cation exchange resins, have polyionic ligands bound to a silica surface. The ligands of the LiChrospher® 1000 SO_3^- column (EM Separations, Gibbstown, New Jersey) used in this example are advertised as containing between 25 and 50 sulfopropyl groups along a polyethylene backbone that is joined at one end to the silica surface.

The TCX chromatogram of a sample of recombinant human DNase run on a LiChrospher® 1000 SO_3^- column is shown in Figure 5. Recombinant human DNase was purified from cultures of Chinese hamster ovary (CHO) cells transformed with DNA encoding human DNase. Shak, et al., Proc. Nat. Acad. Sci. 87:9188-9192 (1990); Shak, et al., International Patent Application Publication No. WO 90/07572 (published 12 July 1990).

The two peaks obtained were collected and subjected to several analyses in order to identify them as the forms of DNase differing only at the residue at amino acid position 74. Figure 6 shows tryptic maps of the two peaks collected from the TCX column, confirming that they are, respectively, the deamidated and non-deamidated forms of human DNase. The tryptic map also reveals that both forms of deamidated DNase (having Asp and iso-Asp at amino acid position 74) are present in the first peak from the TCX separation. Table II shows the specific activities measured for the two peaks, confirming the relationship between deamidation and

specific activity inferred from the correlation shown in Figure 2, and further supporting the identification of the TCX fractions. Activity of the DNase fraction was determined by a methyl green (MG) assay.

TABLE II. ACTIVITIES OF FRACTIONS COLLECTED FROM TCX COLUMN.

MG and ELISA concentrations are the averages of determinations on two samples.

Sample	MG (μg/ml)	ELISA (μg/ml)	Specific Activity
Starting preparation of recombinant human Dnase (load)	8315	7828	1.06
TCX Peak 1 (deamidated)	85.3	119.7	0.71
TCX Peak 2 (non-deamidated)	149.2	99.4	1.50

A mutant form of human DNase, having an Asp residue at amino acid position 74, was produced by site-directed mutagenesis of the DNA encoding native mature human DNase. This mutant coelutes with the first peak obtained in the above chromatography, as shown in Figure 7.

The following is the procedure used to pack the LiChrospher® 1000 SO_3^- tentacle cation exchange resin. Another tentacle cation exchange resin similarly useful for separation of deamidated and non-deamidated forms of human DNase is Fractogel® tentacle cation exchange resin (EM Separations, Gibbstown, New Jersey). LiChrospher and Fractogel are registered trademarks of EM Industries, Inc., Hawthorne, N.Y., or E. Merck, Darmstadt, West Germany. The "strong" forms of the tentacle cation exchange resins (whether LiChrospher or Fractogel), having a SO_3^- functional group, appear at this time to give the best results.

3. HPLC Column Packing Procedure for LiChrospher® 1000 SO_3^- Resin.

a. Materials and Equipment:

1. Superformance glass cartridge 1.0 cm x 5.0 cm bed.
2. Packing Buffer: 10mM sodium acetate, 1mM $CaCl_2$, pH to 4.5 with acetic acid. Filter through a 0.2μ filter.
3. Column packing reservoir with a capacity of 20 ml. (Alltech part # 9501 or equivalent).
4. Empty 4.6mm x 50mm stainless steel column with 0.5 μ cut-off frits
5. HPLC pump capable of maintaining a back pressure of 2000 psi (Waters Model 510 or equivalent).

b. Packing Procedure.

1. De-fine resin:

a) Unpack 1.0 cm x 5.0 cm Superformance glass column (Bed volume = 3.93 ml resin). Resuspend resin to 20 mls in a clear glass, capped vessel with column packing buffer. Slurry into a uniform suspension and divide into 2 x 10 ml aliquots. Add 10 mls of column packing buffer to each aliquot to achieve suspensions of approx. 1.95 mls resin in 20 mls packing buffer.

b) Slurry resin to achieve a uniform suspension. Allow to settle until particles form a solid bed on the bottom of the vessel (2-4 hours). Carefully pour off the supernatant containing fine particles.

c) Add 20 mls. packing buffer to resin and repeat step b). This procedure should be repeated at least four times to assure removal of all fine resin particles.

2. Column Packing:

a) Connect 4.6 mm x 50 mm empty HPLC column to packing reservoir. Slurry resin in 20 mls of packing buffer.

b) Add slurried resin to reservoir and quickly cap. Pump packing buffer at a pressure that does not exceed 2000 psi. Adjust flow rate so that packing pressure remains constant at about 2000 psi and flow for 15 minutes after pressure stabilizes. Remove column and attach top end. Column may be used directly or stored in 0.02% sodium azide.

For most samples, including DNase formulated in 150 mM NaCl, no sample preparation is required prior to injection of the sample onto the column. The column is equilibrated with a pH 4.5 acetate buffer containing calcium ions, the sample is injected, and the column then is eluted with a salt gradient. The following procedure is useful for small-scale separations of deamidated and non-deamidated forms of human DNase. The proportions of the peak areas on the resulting chromatogram are equal to the proportions of deamidated and non-deamidated DNase in the sample.

Step 1. Load sample, containing up to 150 mM NaCl and at a pH up to 9 into autosampler vial. Harvested cell culture fluid samples require adjustment of pH to 4.5 and centrifugation to remove proteins that are insoluble in the buffers used in this procedure.

Step 2. Separate the two forms of DNase by HPLC under the following conditions:
Column: TCX LiChrospher® 1000 SO_3^- repacked into a steel column. Column dimensions of 4.6 x 50 mm and 4.6 x 150 mm have been packed and employed.
Column temperature: ambient.
Eluent A: 10 mM sodium acetate, 1 mM CaCl2, pH 4.5.
Eluent B: 1 M NaCl in buffer A.
Gradient profile:

Time (min)	%A	%B
0	100	0
4	100	0
30	30	70
30.1	5	95
37	5	95

Flow rate: 0.8 ml/min (50 mm column), 0.5 ml/min (150 mm column).
Sample injection volume: up to 250 µl.
Post-run column reequilibration time at 100% A: 20 min.
Autosampler compartment temperature: 5°.
Detection: Absorbance at 280 nm.

Step 3. Integrate chromatogram. Calculate the proportion of deamidated species based on the peak area of the earlier eluting deamidated DNase relative to the total peak area of both forms.

Tentacle cation exchange chromatography also provides a means for separating, at large scale, the deamidated and non-deamidated forms of human DNase. Large scale separations are more conveniently carried out using simplified elution operating conditions than are described above for small-scale analytical separations of the two forms of DNase. Hence, larger scale separations have been carried out on the Fractogel-supported tentacle cation exchanger according to the following pH-elution procedure:

Step 1. Pack 31.6 column (1.6 cm i.d. x 15.7 cm high) with Fractogel EMD SO_3-650M tentacle cation exchange resin (EM Separations, Gibbstown, New Jersey).

Step 2. Diafilter DNase load with equilibration buffer (30 mM sodium acetate (NaAc), 1 mM calcium chloride ($CaCl_2$), 50 mM sodium chloride (NaCl), pH 5). Concentrate by ultrafiltration to volume of 355 mls and concentration of 2.5 mg/ml.

Step 3. Wash column with 2.5 column volumes (CV) of 2% sodium hydroxide (NaOH).

Step 4. Wash column with 2.5 CV of pre-equilibration buffer (300 mM NaAc, 1 M NaCl, pH 5).

Step 5. Wash column with 2.5 CV of equilibration buffer.

Step 6. Load column with 1-1.3 g of diafiltered / ultrafiltered DNase (from Step 2). Begin collecting fractions of column effluent upon commencement of DNase load.

Step 7. Wash column with 5 CV of equilibration buffer.

Step 8. Wash column with 5 CV of pH 5.3 wash buffer (25 mM succinate, 1 mM $CaCl_2$, pH 5.3).

Step 9. Wash column with 10 CV of pH 5.4 wash buffer (25 mM succinate, 1 mM $CaCl_2$, pH 5.4).

Step 10. Wash column with 10 CV of pH 6 wash buffer (25 mM MES, 1 mM $CaCl_2$, pH 6.0).

Step 11. Combine fractions collected during Steps 6-8 to make a pool consisting predominantly of deamidated DNase. Combine fractions collected during Step 10 to make a non-deamidated DNase pool. Fractions collected during Step 9 contain a mixture of the two forms of DNase and may be recycled.

The protocol described above is one example of the use of a tentacle cation exchange resin for a preparative purification of the two forms of recombinant human DNase in a manner that is scaleable to large-scale recovery of purified deamidated and purified non-deamidated DNase.

4. Heparin and Immobilized DNA Analog Chromatography.

In Figure 8 chromatograms are aligned of analyses on a TSK-Heparin column (Toso Haas, Montgomeryville, Pennsylvania) of samples containing either a mixture of deamidated and non-deamidated forms of human DNase, purified deamidated human DNase, or purified non-deamidated human DNase. The TSK-Heparin column was run under the same conditions as described above for running the analytical TCX column. The aligned chromatograms demonstrate that the column of immobilized heparin resolves deamidated and non-deamidated forms of DNase.

As described above, another means of separating the deamidated and non-deamidated forms of DNase is to employ a column containing an immobilized analog of DNA that is resistant to hydrolysis by DNase. One example of this approach to an immobilized DNA analog column involved the synthesis of the phosphorothioate oligonucleotide 5'-GCGCGCGCGCGCGCGCGCGC-NH_2-3'. This self-complementary sequence can be annealed into a double-stranded form, and coupled to a Rainin Hydropore-EP column (Rainin Co., Woburn, Massachusetts). Figure 9 shows aligned chromatograms of the analyses on this column of samples containing either a mixture of deamidated and non-deamidated forms of human DNase, purified deamidated human DNase, purified non-deamidated human DNase, or purified mutant human DNase having an aspartic acid residue (rather than an asparagine residue) at amino acid position 74. The column was run for these analyses in a buffer containing 1 mM calcium chloride, 5 mM MES at a pH of 6, and eluted with a linear gradient in salt concentration to 1 M sodium chloride over 20 minutes at a flow rate of 1 ml/min. As shown in Figure 9, under these conditions deamidated and non-deamidated DNase forms are partially separated from each other. In addition, the two isomeric forms of deamidated DNase, that differ at amino acid position 74 of the DNase sequence by having either aspartic acid or iso-aspartic acid at this position, are also resolved by this column. Thus an additional benefit of this chromatographic method is that it allows the isolation of the two isomers that arise on deamidation of human DNase.

5. Enzymatic Activity of Deamidated Human DNase and Non-deamidated Human DNase.

Several analytical methods have been used to examine the effect of deamidation on the enzymatic activity of human DNase. Purified deamidated human DNase and purified non-deamidated human DNase for use in these studies were prepared by TCX chromatography, as described above.

In one method for determination of DNase enzymatic activity, synthetic double stranded DNA, 25 base pairs in length, was labeled with dinitrophenol (DNP) on one end and with biotin on the other end. Hydrolysis of the substrate by DNase was detected by capture of the reaction products on microtiter plate wells coated with antibody to DNP and by quantitation of the intact probe with streptavidin-horseradish peroxidase. The specific activity of stability samples was correlated (r^2=0.613;n=5) with the extent of DNase deamidation (range 27% - 93%). Extrapolation of the least squares linear equation provided an estimate that the specific activity of deamidated human DNase was approximately 77% lower than that of non-deamidated human DNase.

Another method for determination of DNase enzymatic activity involved hydrolysis of the chromogenic substrate p-nitrophenyl phenylphosphonate (PNPP) as described by Liao, et al., Biochem. J. 255: 781-787 (1988). The kinetics of PNPP hydrolysis by human DNase are sigmoidal and were fit to the Hill equation by nonlinear regression. By this method the V_{max} of fully deamidated human DNase was determined to be 77% lower than that of non-deamidated human DNase. The substrate concentration for half maximal activity ($S_{0.5}$) did not differ significantly for the deamidated and non-deamidated human DNase samples.

Another method for determination of DNase enzymatic activity is the assay described by Kunitz, J. Gen. Physiol. 33:349 (1950), preferably modified such that the enzymatic reaction is carried out at about pH 7.0 - 7.5. By this method, the enzymatic activity of deamidated human DNase also was determined to be lower than that of non-deamidated human DNase.

6. In Vitro Storage of Human DNase.

Human DNase purified from recombinant CHO cells was dissolved at a concentration of 4 mg/ ml in an unbuffered aqueous solution of 150 mM NaCl and 1 mM $CaCl_2$. Samples of the resulting DNase solution were then placed into glass and plastic vials. Two different types of plastic vials were used, one being made of Dupont 20 plastic resin (manufactured by E.I. du Pont de Nemours & Co., Inc., Wilmington, Delaware USA), and the other being made of Escorene plastic resin (manufactured by Exxon Corp.). Both of those plastics are low density polyethylene, but containers formulated with other plastics, such as polypropylene, polystyrene, or other polyolefins also may be used. The vials containing the DNase solution were stored at either -70° C, 2-8° C, or 25° C. Initially, about 60% - 65% of the DNase in the solutions was deamidated.

The DNase solutions in the vials were assayed at several times after initial storage to determine the extent of deamidation of the DNase. The results of those assays are shown in Table III.

TABLE III. % DEAMIDATION OF RECOMBINANT HUMAN DNASE STORED IN GLASS AND PLASTIC VIALS.

Sample	Day	-70°C	2-8°C	25°C
Glass	83	66	66	78
	174	63	66	81
Dupont 20	83	65	66	71
	174	63	63	70
Escorene	83	65	66	71
	174	64	62	70

After 83 and 174 days storage at -70° C or 2-8°C, no difference was found in the amount of deamidated DNase in the plastic vials and the amount of deamidated DNase in the glass vials. In each such case, approximately 64% (+/- 2%) of the DNase in the vials was deamidated DNase.

Unexpectedly, however, after 83 or 174 days storage at 25° C, there was a difference in the amount of deamidated DNase in the plastic vials and the amount of deamidated DNase in the glass vials. Significantly less deamidated DNase was present in the plastic vials. In particular, after 83 days storage at 25° C, 78% of the DNase in the glass vials was deamidated DNase, whereas only about 70% of the DNase in the plastic vials was deamidated DNase. After 174 days storage at 25° C, 81% of the DNase in the glass vials was deamidated DNase, whereas only about 71% of the DNase in the plastic vials was deamidated DNase.

Without limiting the invention to any particular mechanism or theory of operation, it may be that the differences in deamidation of DNase in plastic and glass vials may be a consequence of differences in the pH of the solutions in the vials. Initially, the pH of the DNase solution in the glass vials was slightly higher than that in the plastic vials (approximately pH 6.7 and approximately pH 6.5, respectively). The pH of the DNase solution in the glass vials continued to increase slightly over time (to approximately pH 6.9 after 83 days storage at 25° C, and approximately pH 7.0 after 174 days storage at 25°C), perhaps as consequence of silicates or ions from the glass surface dissolving in the solution. At higher pH, the rate of deamidation of human DNase is increased. Since it was not appreciated that deamidation of human DNase occurs at elevated pH, it is an embodiment of this invention to formulate

and/or store human DNase in solutions having acidic pH, typically at about pH 4.5 - 6.8 and most preferably at about pH 5.0 - 6.8.

Thus, a significant improvement in the stability of human DNase in solution is obtained by placing such DNase solution in plastic vials rather than glass vials, with apparently less deamidation of the DNase occurring over time in the plastic vials than in the glass vials. This finding may be especially relevant to the choice of packaging of human DNase for therapeutic use, where it is especially desirable that the human DNase be capable of storage for extended periods of time without significant loss of enzymatic activity. Of course, glass vials with non-glass coatings, for example, plastic linings, would be equally useful. What is important is to avoid storing DNase in contact with glass, especially for storage exceeding about 15 - 30 days.

General Remarks

The foregoing description details specific methods which can be employed to practice the present invention. Having detailed specific methods used to identify, characterize, separate and use the pure deamidated and non-deamidated human DNase hereof, and further disclosure as to specific model systems pertaining thereto, those skilled in the art will well enough know how to devise alternative reliable methods for arriving at the same information in using the fruits of the present invention, Thus, however detailed the forgoing may appear in text, it should not be construed as limiting the overall scope hereof; rather, the ambit of the present invention is to be determined only by the lawful construction of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Genentech, Inc.

(ii) TITLE OF INVENTION: PURIFIED FORMS OF DNase

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 460 Point San Bruno Blvd
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: patin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Johnston, Sean A.
(B) REGISTRATION NUMBER: 35,910
(C) REFERENCE/DOCKET NUMBER: 747

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415/225-3562
(B) TELEFAX: 415/952-9881
(C) TELEX: 910/371-7168

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 346 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Ser Cys Thr Gly Ser Ala Leu Lys Cys Phe Phe Arg Asp Leu Ser
 1               5                  10                  15

Ser Xaa Thr Thr Phe Phe Ser Leu Ser Ser Lys Arg Arg Lys Leu
            20                  25                  30

Ser Ser Lys Asp Ile Pro Asp Ser Xaa Gln His Ser Arg His Leu
        35                  40                  45

Xaa Gly His His His His Leu Arg Met Arg Gly Met Lys Leu Leu
    50                  55                  60

Gly Ala Leu Leu Ala Leu Ala Ala Leu Leu Gln Gly Ala Val Ser
65                  70                  75

Leu Lys Ile Ala Ala Phe Asn Ile Gln Thr Phe Gly Glu Thr Lys
```

80 85 90

Met Ser Asn Ala Thr Leu Val Ser Tyr Ile Val Gln Ile Leu Ser
95 100 105

Arg Tyr Asp Ile Ala Leu Val Gln Glu Val Arg Asp Ser His Leu
110 115 120

Thr Ala Val Gly Lys Leu Leu Asp Asn Leu Asn Gln Asp Ala Pro
125 130 135

Asp Thr Tyr His Tyr Val Val Ser Glu Pro Leu Gly Arg Asn Ser
140 145 150

Tyr Lys Glu Arg Tyr Leu Phe Val Tyr Arg Pro Asp Gln Val Ser
155 160 165

Ala Val Asp Ser Tyr Tyr Tyr Asp Asp Gly Cys Glu Pro Cys Gly
170 175 180

Asn Asp Thr Phe Asn Arg Glu Pro Ala Ile Val Arg Phe Phe Ser
185 190 195

Arg Phe Thr Glu Val Arg Glu Phe Ala Ile Val Pro Leu His Ala
200 205 210

Ala Pro Gly Asp Ala Val Ala Glu Ile Asp Ala Leu Tyr Asp Val
215 220 225

Tyr Leu Asp Val Gln Glu Lys Trp Gly Leu Glu Asp Val Met Leu
230 235 240

Met Gly Asp Phe Asn Ala Gly Cys Ser Tyr Val Arg Pro Ser Gln
245 250 255

Trp Ser Ser Ile Arg Leu Trp Thr Ser Pro Thr Phe Gln Trp Leu
260 265 270

Ile Pro Asp Ser Ala Asp Thr Thr Ala Thr Pro Thr His Cys Ala
275 280 285

Tyr Asp Arg Ile Val Val Ala Gly Met Leu Leu Arg Gly Ala Val
290 295 300

Val Pro Asp Ser Ala Leu Pro Phe Asn Phe Gln Ala Ala Tyr Gly
305 310 315

Leu Ser Asp Gln Leu Ala Gln Ala Ile Ser Asp His Tyr Pro Val
320 325 330

Glu Val Met Leu Lys Xaa Ala Ala Pro Pro His Thr Ser Xaa Thr
335 340 345

Ala
346

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1039 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCCTGCACAG GCAGTGCCTT GAAGTGCTTC TTCAGAGACC TTTCTTCATA 50

GACTACTTTT TTTTCTTTAA GCAGCAAAAG GAGAAAATTG TCATCAAAGG 100

ATATTCCAGA TTCTTGACAG CATTCTCGTC ATCTCTGAGG ACATCACCAT 150

CATCTCAGGA TGAGGGGCAT GAAGCTGCTG GGGGCGCTGC TGGCACTGGC 200

GGCCCTACTG CAGGGGGCCG TGTCCCTGAA GATCGCAGCC TTCAACATCC 250

AGACATTTGG GGAGACCAAG ATGTCCAATG CCACCCTCGT CAGCTACATT 300

GTGCAGATCC TGAGCCGCTA TGACATCGCC CTGGTCCAGG AGGTCAGAGA 350

CAGCCACCTG ACTGCCGTGG GGAAGCTGCT GGACAACCTC AATCAGGATG 400

CACCAGACAC CTATCACTAC GTGGTCAGTG AGCCACTGGG ACGGAACAGC 450

TATAAGGAGC GCTACCTGTT CGTGTACAGG CCTGACCAGG TGTCTGCGGT 500

GGACAGCTAC TACTACGATG ATGGCTGCGA GCCCTGCGGG AACGACACCT 550

TCAACCGAGA GCCAGCCATT GTCAGGTTCT TCTCCCGGTT CACAGAGGTC 600

AGGGAGTTTG CCATTGTTCC CCTGCATGCG GCCCCGGGGG ACGCAGTAGC 650

CGAGATCGAC GCTCTCTATG ACGTCTACCT GGATGTCCAA GAGAAATGGG 700

GCTTGGAGGA CGTCATGTTG ATGGGCGACT TCAATGCGGG CTGCAGCTAT 750

GTGAGACCCT CCCAGTGGTC ATCCATCCGC CTGTGGACAA GCCCCACCTT 800

CCAGTGGCTG ATCCCCGACA GCGCTGACAC CACAGCTACA CCCACGCACT 850

GTGCCTATGA CAGGATCGTG GTTGCAGGGA TGCTGCTCCG AGGCGCCGTT 900

GTTCCCGACT CGGCTCTTCC CTTTAACTTC CAGGCTGCCT ATGGCCTGAG 950

TGACCAACTG GCCCAAGCCA TCAGTGACCA CTATCCAGTG GAGGTGATGC 1000

TGAAGTGAGC AGCCCCTCCC CACACCAGTT GAACTGCAG 1039

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ile Ala Ala Phe Asn Ile Gln Thr Phe Gly Glu Thr Lys
1 5 10 13

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ser Asn Ala Thr Leu Val Ser Tyr Ile Val Gln Ile Leu Ser
1 5 10 15

Arg
16

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Tyr Asp Ile Ala Leu Val Gln Glu Val Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Asp Ser His Leu Thr Ala Val Gly Lys
1 5 9

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu Leu Asp Asn Leu Asn Gln Asp Ala Pro Asp Thr Tyr His Tyr
1 5 10 15

Val Val Ser Glu Pro Leu Gly Arg
20 23

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Asn Ser Tyr Lys
1 4

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Tyr Leu Phe Val Tyr Arg Pro Asp Gln Val Ser Ala Val Asp Ser
1 5 10 15

Tyr Tyr Tyr Asp Asp Gly Cys Glu Pro Cys Gly Asn Asp Thr Phe
20 25 30

Asn Arg
32

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Glu Pro Ala Ile Val Arg
1 5 6

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Phe Phe Ser Arg
1 4

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Phe Thr Glu Val Arg
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Glu Phe Ala Ile Val Pro Leu His Ala Ala Pro Gly Asp Ala Val
1 5 10 15

Ala Glu Ile Asp Ala Leu Tyr Asp Val Tyr Leu Asp Val Gln Glu
20 25 30

Lys
31

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Trp Gly Leu Glu Asp Val Met Leu Met Gly Asp Phe Asn Ala Gly
1 5 10 15

Cys Ser Tyr Val Arg Pro Ser Gln Trp Ser Ser Ile Arg
20 25 28

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Leu Trp Thr Ser Pro Thr Phe Gln Trp Leu Ile Pro Asp Ser Ala
1 5 10 15

Asp Thr Thr Ala Thr Pro Thr His Cys Ala Tyr Asp Arg
20 25 28

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ile Val Val Ala Gly Met Leu Leu Arg
1 5 9

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Gly Ala Val Val Pro Asp Ser Ala Leu Pro Phe Asn Phe Gln Ala
1 5 10 15

Ala Tyr Gly Leu Ser Asp Gln Leu Ala Gln Ala Ile Ser Asp His
20 25 30

Tyr Pro Val Glu Val Met Leu Lys
35 38

CLAIMS

What is claimed is:

1. A process comprising separating deamidated and non-deamidated human DNase from a mixture thereof.

2. A process according to claim 1 which employs a tentacle cation exchange resin.

3. A process according to claim 1 which employs an immobilized heparin resin.

4. A process according to claim 1 which employs an immobilized non-hydrolyzable DNA analog resin.

5. Purified deamidated human DNase.

6. Purified non-deamidated human DNase.

7. A pharmaceutical composition consisting of deamidated human DNase as the active principle and optionally a pharmaceutically acceptable excipient.

8. A pharmaceutical composition consisting of non-deamidated human DNase as the active principle and optionally a pharmaceutically acceptable excipient.

9. A pharmaceutical composition according to claim 8 wherein the excipient is sterile water.

10. A pharmaceutical composition according to claim 8 wherein the excipient is a sterile unbuffered aqueous solution at about pH 4.5 - 6.8.

11. A pharmaceutical composition according to claim 8 wherein said composition is in an aerosol form.

12. A pharmaceutical composition according to claim 9 disposed in contact with a container fabricated of other than glass.

13. A pharmaceutical composition according to claim 10 disposed in contact with a container fabricated of other than glass.

14. A pharmaceutical composition comprising non-deamidated human DNase in a plastic vial.

15. A pharmaceutical composition according to claim 14 that is substantially free of deamidated human DNase.

16. A method for storing human DNase comprising preparing a composition comprising non-deamidated human DNase in an aqueous solution having a pH of about 4.5 to 6.8 and storing the composition for greater than about three weeks.

17. A method for the treatment of a patient having an accumulation of purulent material comprising administering purified non-deamidated human DNase to the patient in an amount therapeutically effective to reduce the viscoelasticity of the material.

18. A method according to claim 17 wherein said non-deamidated human DNase is substantially free of proteases.

19. A method for the treatment of a patient having cystic fibrosis comprising administering to such patient a therapeutically effective amount of purified non-deamidated human DNase.

Fig. 1.

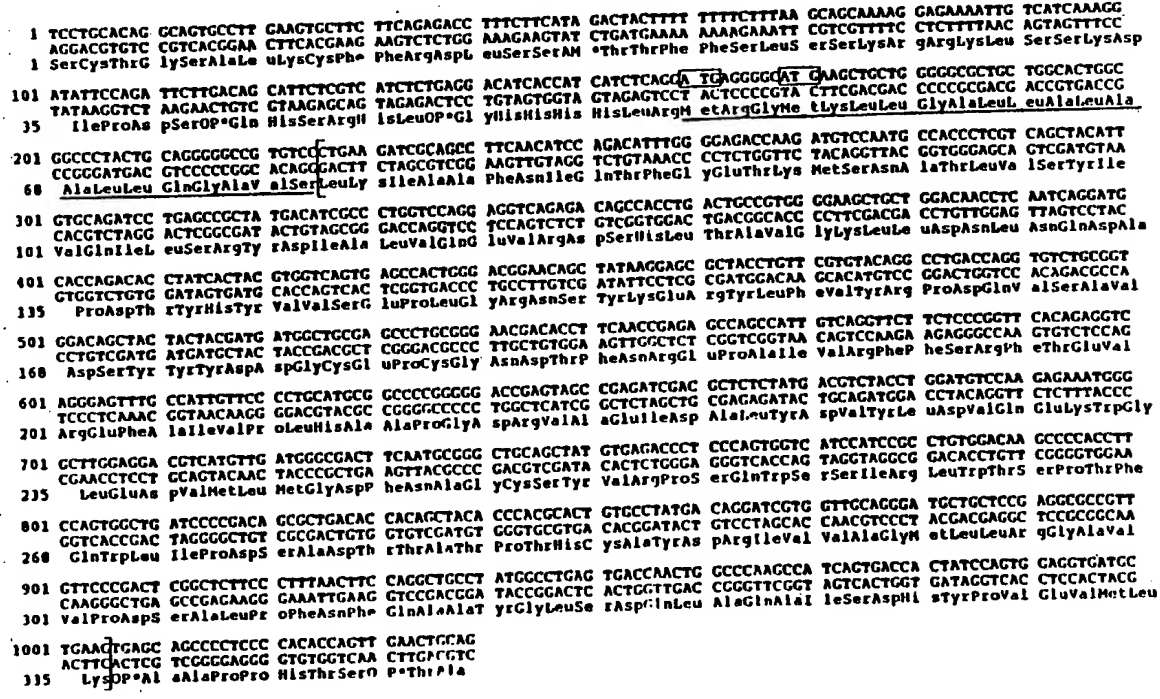
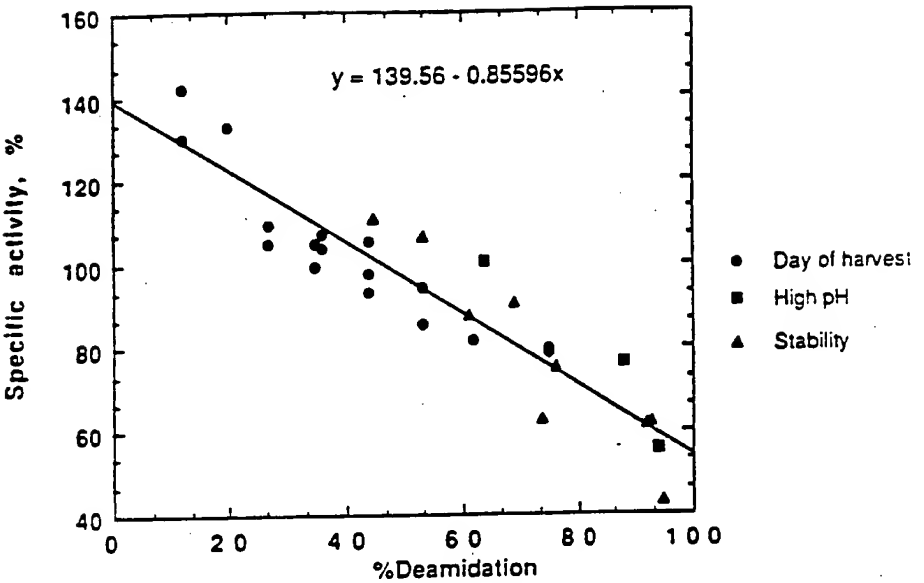


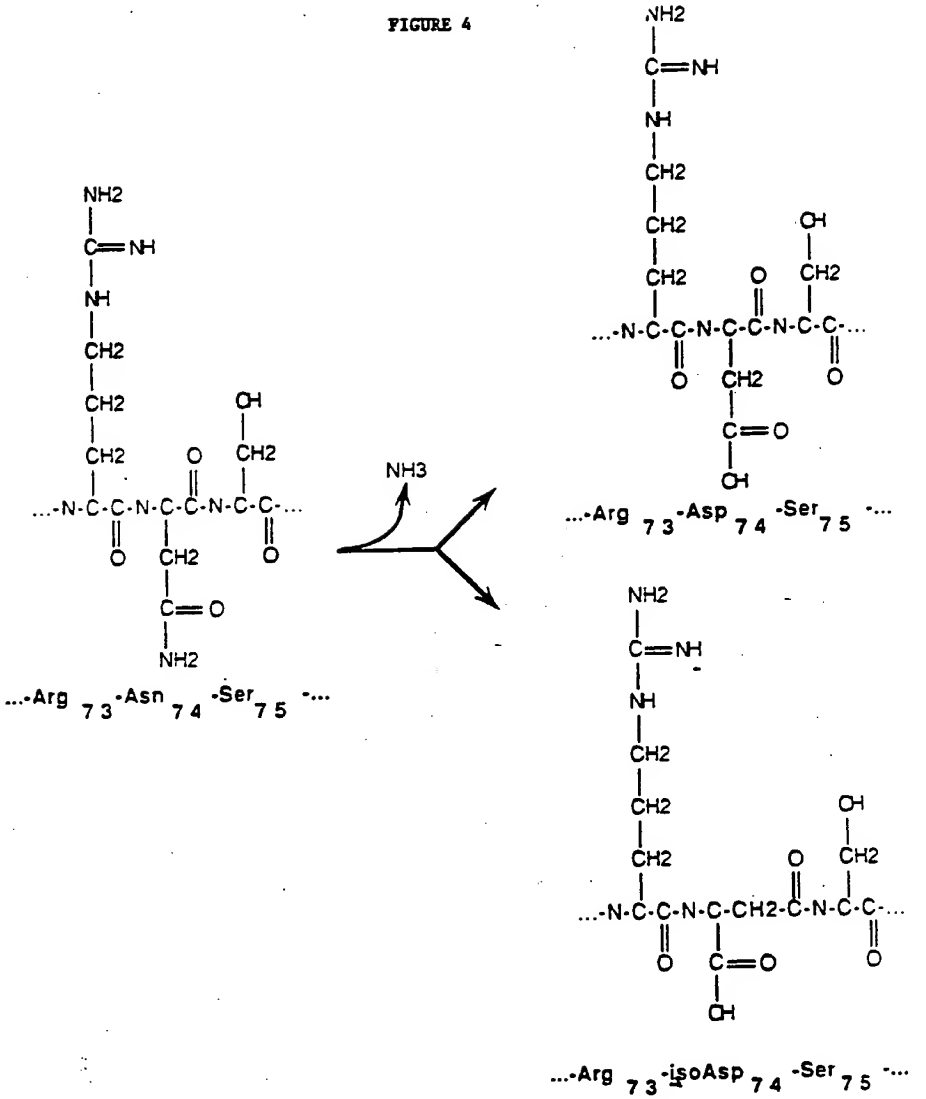
FIGURE 2


y = 139.56 - 0.85596x
Specific activity, %
%Deamidation
160
140
120
100
80
60
40
0
20
40
60
80
100
Day of harvest
High pH
Stability

mAU
2500
2000
1500
1000
500
0
Void Vol.
T1
T7(D)T7
T7-8 (D)T7-8
T12,T5
T11
T4
T6-7-8
T6-7,T2
T6
T16
T9-10
T14-15
T13,T17
undig.
10
20
30
40
50
60
Time (min.)

FIGURE 3

FIGURE 4


NH3
....-Arg 73 -Asn 74 -Ser 75 -....
....-Arg 73 -Asp 74 -Ser 75 -....
....-Arg 73 -isoAsp 74 -Ser 75 -....

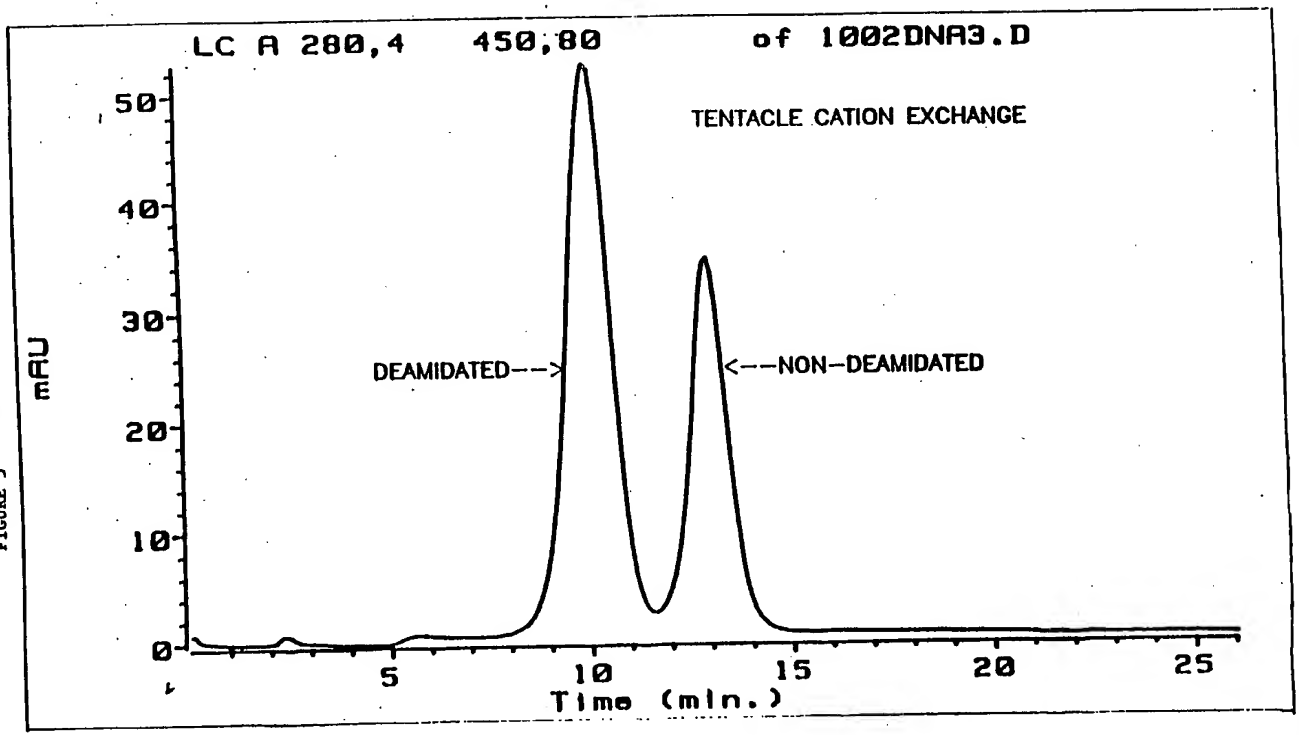

LC A 280,4 450,80 of 1002DNA3.D
TENTACLE CATION EXCHANGE
mAU
50
40
30
20
10
0
DEAMIDATED-->
<--NON-DEAMIDATED
5
10
15
20
25
Time (min.)

FIGURE 5

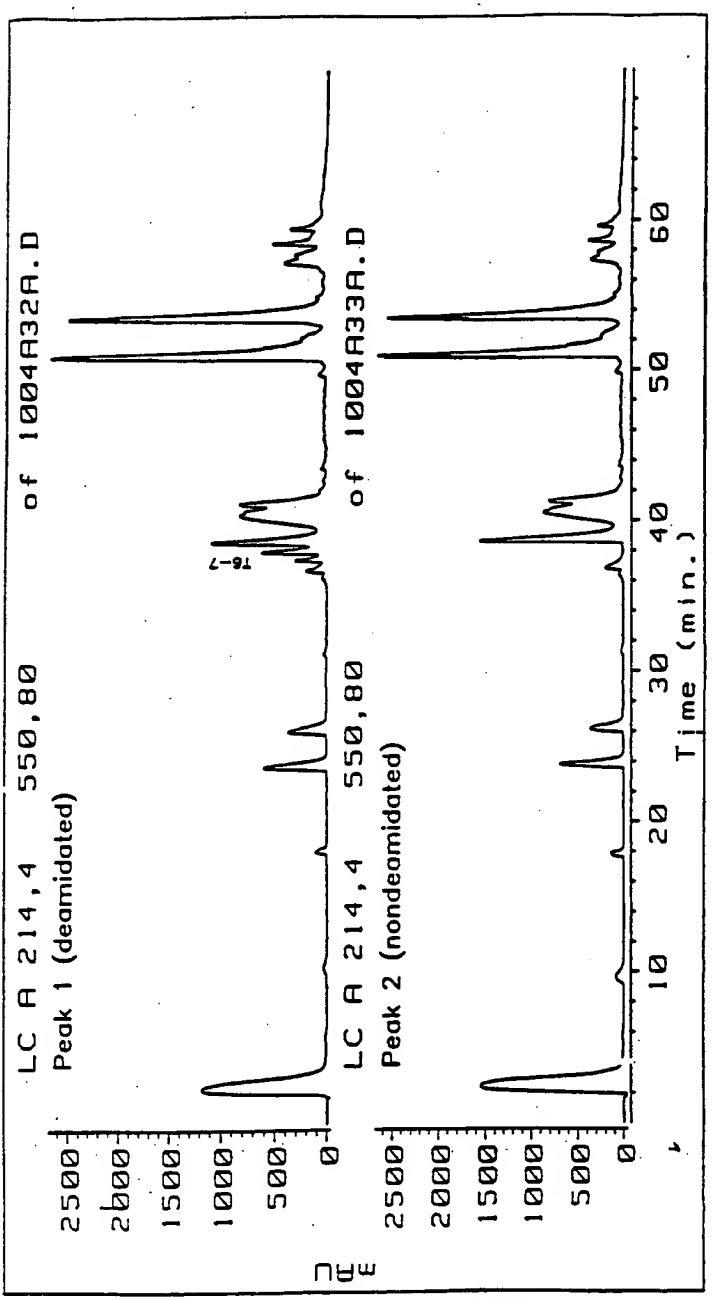

LC A 214,4 550,80 of 1004A32A.D
Peak 1 (deamidated)
LC A 214,4 550,80 of 1004A33A.D
Peak 2 (nondeamidated)
mAU
2500
2000
1500
1000
500
0
10
20
30
40
50
60
Time (min.)
T6-7

FIGURE 6

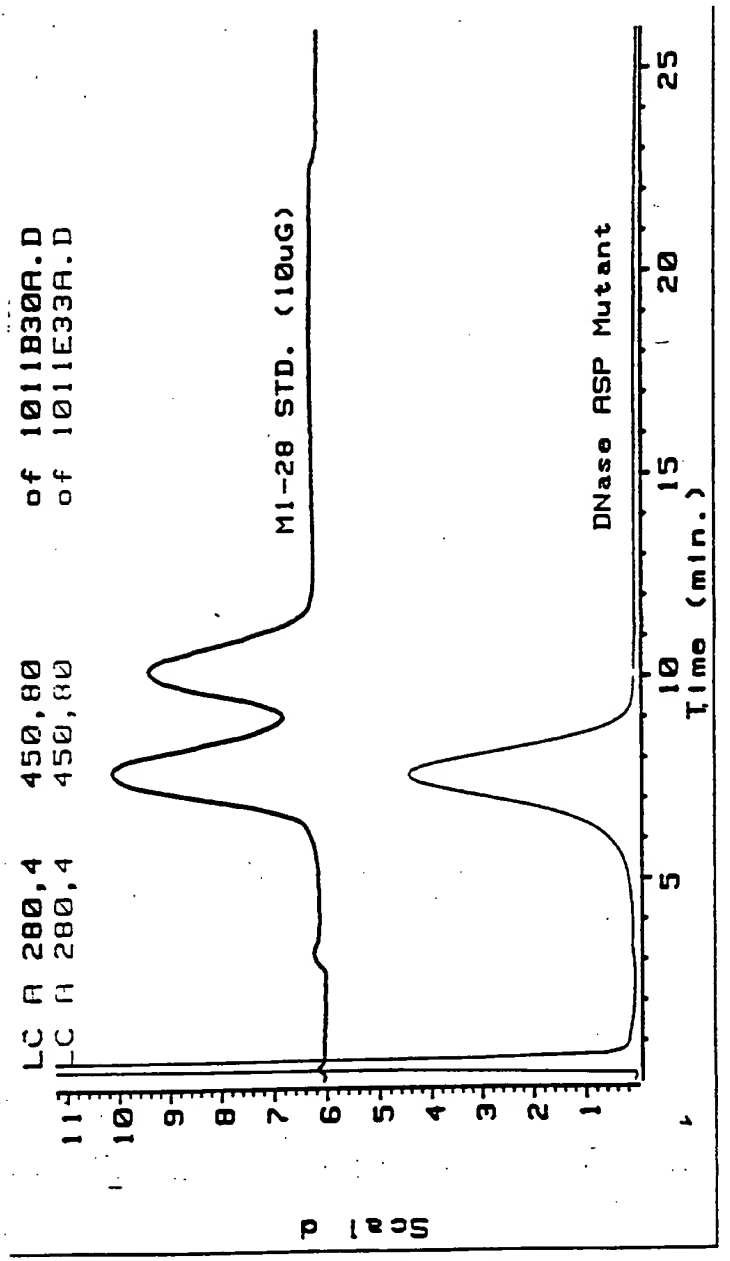
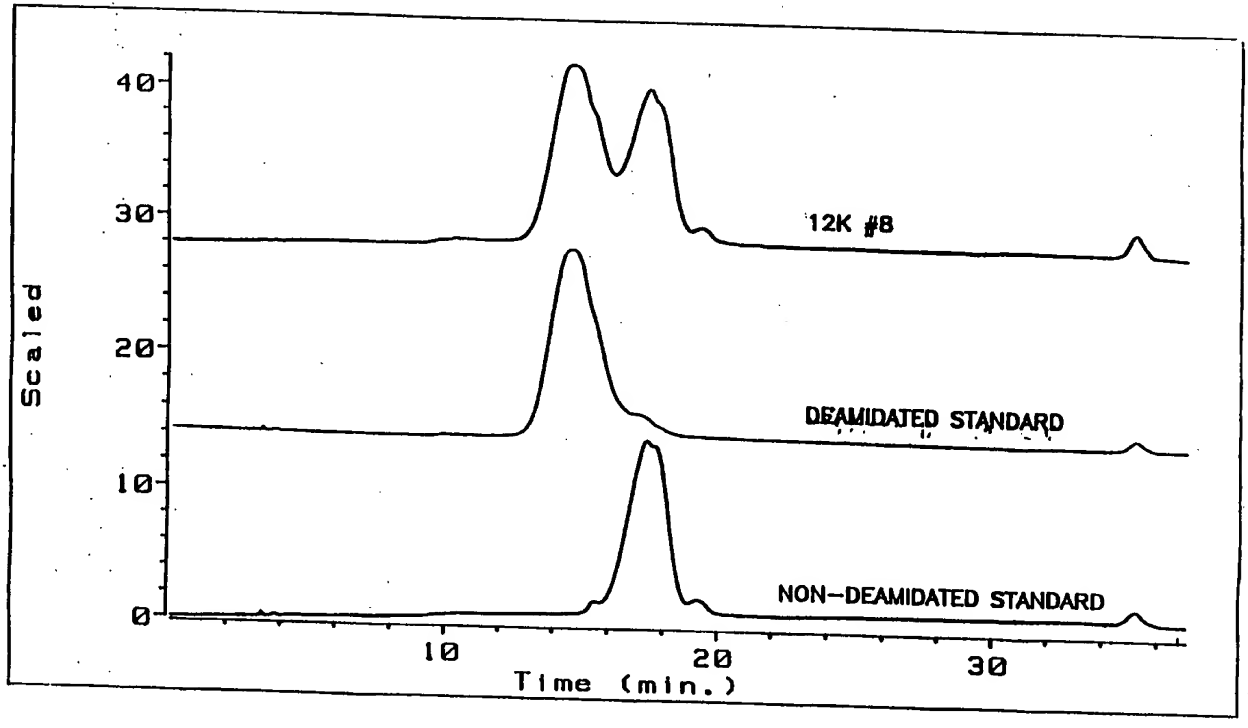

LC A 280,4 450,80 of 1011B30A.D
LC A 280,4 450,80 of 1011E33A.D
M1-28 STD. (10uG)
DNase ASP Mutant
Time (min.)
Scale
11
10
9
8
7
6
5
4
3
2
1
5
10
15
20
25

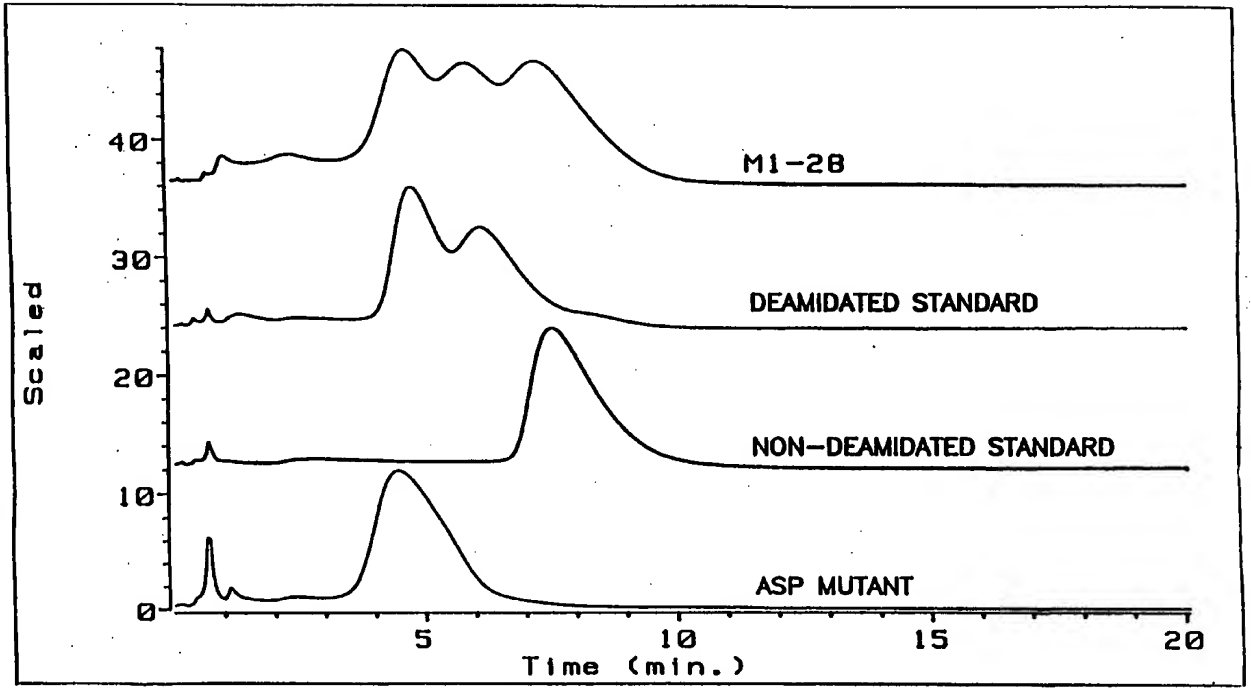
FIGURE 7 6/7

FIGURE 8



TSK HEPARIN 7.5 X 75mm A:1mM CaCl2, 10mM ACETATE pH 4.5
B: A+1M NaCl 0%B,4mn->58%B,26min->95%B,0.1mn,7min ISOC
1.0 mL/min

FIGURE 9



GC OLIGO COLUMN 4.6X50mm A:1mM CaCl2 5mM MES pH 6.0
B:A +1M NaCl 0%B-100%B,20min 1mL/min

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/05136

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N9/22; C07K3/20; A61K37/54

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	WO,A,9 007 572 (GENENTECH, INC.) 12 July 1990 cited in the application ---	1
A	BIOCHEMISTRY. vol. 30, no. 16, 23 April 1991, EASTON, PA US pages 3916 - 3922 GLEN TESHIMA ET AL. 'Deamidation of soluble CD4 at Asparagine-52 results in reduced binding capacity for the HIV-1 envelope glycoprotein gp120' see page 3919, left column, paragraph 4 - page 3920, left column, paragraph 1 --- -/--	1

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 SEPTEMBER 1993	23.09.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MONTERO LOPEZ B.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	JOURNAL OF CHROMATOGRAPHY vol. 480, 1989, AMSTERDAM NL pages 379 - 391 JOHN FRENZ ET AL. 'Characterization of human growth hormone by capillary electrophoresis' see page 379, paragraph 1 - page 380, paragraph 1 see page 383, paragraph 1 - page 385, paragraph 1 see page 390, paragraph 2	1
P,O, X	16th International symposium on column liquid chromatography Baltimore, MD June, 14-19, 1992 & JOURNAL OF CHROMATOGRAPHY vol. 634, 1993, AMSTERDAM NL pages 229 - 239 J. CACIA ET AL. 'Protein sorting by high-performance liquid chromatography. I. Biomimetic interaction chromatography of recombinant human deoxyribonuclease I on polyionic stationary phases' see abstract see page 230, right column, paragraph 2 - page 231, left column, paragraph 1 see page 234, right column, paragraph 2 - page 238, left column, paragraph 1	1-6

INTERNATIONAL SEARCH REPORT

Int tional application No.
PCT/US93/05136

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 17-19 are directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the compound.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9305136
SA 75127

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 06/09/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9007572	12-07-90	AU-B- 630658	05-11-92
		AU-A- 4826590	01-08-90
		CA-A- 2006473	23-06-90
		EP-A- 0449968	09-10-91
		JP-T- 4502406	07-05-92